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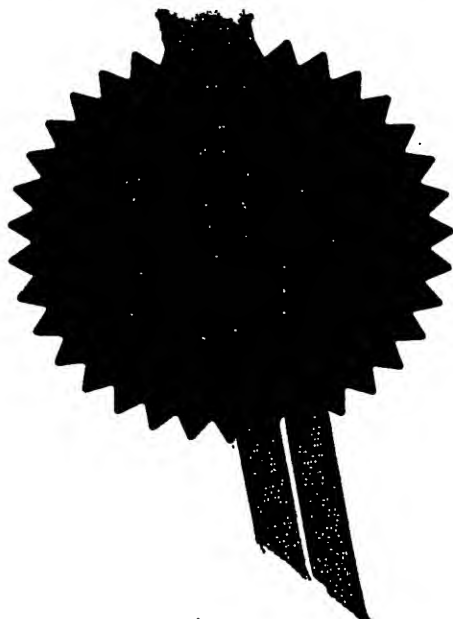
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Patents ADP number (if you know it) 08608812001

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4. Title of the invention

VESICLE FILTRATION

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VESICLE FILTRATION

All documents cited herein are incorporated by reference in their entirety.

TECHNICAL FIELD

This invention is in the field of vesicle preparation for immunisation purposes.

5 BACKGROUND ART

One of the various approaches to immunising against *N.meningitidis* infection is to use outer membrane vesicles (OMVs). An efficacious OMV vaccine against serogroup B has been produced by the Norwegian National Institute of Public Health [e.g. ref. 1] but, although this vaccine is safe and prevents NmB disease, its efficacy is limited to the strain used to make the vaccine.

10 The 'RIVM' vaccine is based on vesicles containing six different PorA subtypes and has been shown to be immunogenic in children in phase II clinical trials [2].

References 3 & 4 disclose a vaccine against different pathogenic serotypes of serogroup meningococcus based on OMVs which retain a protein complex of 65-kDa. Reference 5 discloses a vaccine comprising OMVs from genetically-engineered meningococcal strains, with the OMVs
15 comprising: at least one Class 1 outer-membrane protein (OMP) but not comprising a Class 2/3 OMP. Reference 6 discloses OMVs comprising OMPs which have mutations in their surface loops. Reference 7 discloses compositions comprising OMVs supplemented with transferrin binding proteins (e.g. TbpA and TbpB) and/or Cu,Zn-superoxide dismutase. Reference 8 discloses compositions comprising OMVs supplemented by various proteins. References 9 & 10 also describe
20 OMV preparations from meningococcus.

Reference 11 discloses a process for preparing OMV-based vaccines, particularly for serogroup A meningococcus, comprising the following 10 steps: (a) cultivating bacterial cells; (b) concentrating the cultivated cells from step (a); (c) treating the cells with a bile acid salt detergent at a pH sufficiently high not to precipitate the detergent, for inactivating the bacteria, disrupting the outer
25 membrane of the bacteria and forming vesicles of the outer membrane of the bacteria, said vesicles comprising outer membrane components mainly presented in their native form; (d) centrifuging the composition from step (c) at 10,000-20,000 x g for about 1 to 2 hours to separate the outer membrane vesicles from the treated cells and cell debris, and collecting the supernatant; (e) performing a high speed centrifugation of the supernatant from step (d) and collecting the outer membrane vesicles in a
30 pellet; (f) re-dispersing the pellet from step (e) in a buffer by stirring at ambient temperature; (g) performing a second high speed centrifugation in accordance with step (e), collecting the outer membrane vesicles in a pellet; (h) re-dispersing the pellet from step (g) in an aqueous medium containing a stabilising agent by stirring at ambient temperature; (i) performing a step-wise sterile filtration through at least two filters of decreasing pore size of the re-dispersed composition from step
35 (h), ending with a filter of pore-size of about 0.2µm; and (j) optionally including the composition from step (i) in a pharmaceutically acceptable carrier and/or adjuvant composition.

It is an object of the present invention to provide an improved process for preparing OMVs for use in vaccines, in particular a process which can prepare a greater quantity of OMVs in a shorter time, and particularly a process suitable for industrial-scale use.

DISCLOSURE OF THE INVENTION

5 The invention is based on the finding that, compared to the centrifugation used in step (e) of the process of reference 11, ultrafiltration allows much larger amounts of OMV-containing supernatant to be processed in a much shorter time (typically >15 litres in 4 hours, compared to <1.5 litres in 10 hours). As well allowing step (e) to be performed more quickly, the use of ultrafiltration allows step (f) to be avoided because the OMVs remain in suspension.

10 Thus the invention provides a process for preparing bacterial OMVs, comprising a step of ultrafiltration. The ultrafiltration step is performed on an aqueous suspension of OMVs after they have been prepared from bacteria and the OMVs remain in suspension after the ultrafiltration step.

The invention also provides, in a process for preparing OMVs from a bacterium, the improvement consisting of the use of ultrafiltration of an OMV suspension in place of a step of centrifugation.

15 The invention also provides a process for purifying bacterial OMVs, wherein the process does not include a centrifugation step in which the OMVs are pelleted, particularly a centrifugation step performed on crude OMVs.

The ultrafiltration step

20 Ultrafiltration is a separation process whereby solvent is removed from a solution (including a colloidal solution) or a suspension by forcing it to flow through a membrane by the application of a hydraulic pressure. Components in the solution which are significantly larger than the solvent cannot pass through the membrane. Ultrafiltration therefore separates components based on size.

25 The ultrafiltration step preferably results in diafiltration of the solution. In diafiltration, solvent and/or microsolute (e.g. salts) which are removed during ultrafiltration are replaced by new solvent and microsolute. In general, removal and replacement occur at the same rate and the volume of the solution is thus kept constant. The overall effect of the process is therefore the replacement of original solvent/microsolute with new solvent/microsolute. The process of the invention may thus include a step of diafiltration.

30 The ultrafiltration is preferably cross-flow or tangential flow ultrafiltration, in which the solution flows substantially parallel to the membrane surface, rather than flowing perpendicular to the surface as in ordinary filtration.

Preferred membranes for use in the ultrafiltration step have a cut-off of about 300kDa.

Membranes may be made from any suitable material e.g. polyethersulphone.

Pre-ultrafiltration steps

Prior to the ultrafiltration step, the process of the invention will typically comprise an initial step of cultivating bacterial cells (*e.g.* in broth or in solid medium culture), optionally followed by a step of collecting and/or concentrating the cultivated cells (*e.g.* by filtration or by a low-speed centrifugation to pellet the cells). However, the invention may be performed on bacteria which have already been cultured and/or harvested separately.

The ultrafiltration step is performed on an aqueous suspension of OMVs after they have been prepared from bacteria. Prior to ultrafiltration, the process may therefore comprise a step of OMV preparation in which cells are treated to disrupt their outer membranes. The preparation of OMVs from meningococcus is well-known in the art. Methods for obtaining suitable preparations are disclosed in, for instance, references 1 to 25. Techniques for forming OMVs include treating bacteria with a bile acid salt detergent (*e.g.* salts of lithocholic acid, chenodeoxycholic acid, ursodeoxycholic acid, deoxycholic acid, cholic acid, ursocholic acid, *etc.*, with sodium deoxycholate [26 & 27] being preferred for treating *Neisseria*) at a pH sufficiently high not to precipitate the detergent [11]. Other techniques may be performed substantially in the absence of detergent [28] using techniques such as sonication, homogenisation, microfluidisation, cavitation, osmotic shock, grinding, French press, blending, *etc.*

After OMV formation and prior to ultrafiltration, the OMVs are preferably separated from bacterial cells and cell debris. Separation can conveniently be achieved by centrifugation (*e.g.* at 10,000-20,000 x g for about 1 to 2 hours). OMVs remain in the supernatant and can then be subjected to ultrafiltration according to the invention, rather than to ultracentrifugation as in the prior art. Other methods for separating outer membrane fractions from cytoplasmic molecules may involve filtration (*e.g.* cross flow filtration), differential precipitation or aggregation of outer membranes and/or OMVs, affinity separation methods using ligands that specifically recognize outer membrane molecules, *etc.* Use of a closed filtration system may be preferred to avoid open handling of infectious bacteria.

In order to preserve the native conformation of proteins and other labile outer membrane antigens, mild conditions will generally be selected for preparation of OMVs. Heat inactivation of bacteria (*e.g.* at 56°C or higher) is thus preferentially avoided, as is solvent denaturation.

Post-ultrafiltration steps

After the ultrafiltration step, the OMVs may be further treated.

For example, the OMVs may be sterilised. Sterilisation is preferably a final step before packaging as a pharmaceutical, and can conveniently be achieved by filter sterilisation. Although OMVs will pass through a standard 0.22µm filters, these can rapidly become clogged by other material, and so it is preferred to perform sequential steps of filter sterilisation through a series of filters of decreasing pore size, finishing with a standard sterilisation filter (*e.g.* a 0.22µm filter). Examples of preceding

filters would be those with pore size of 0.8 μ m, 0.45 μ m, etc. Filter sterilisation advantageously occurs at ambient temperature or above, rather than at refrigeration temperatures. Vesicle flexibility is higher at ambient temperature and larger vesicles (~0.2 μ m) can thus pass through a 0.22 μ m filter more easily, giving less clogging of filters.

- 5 The OMVs may also be centrifuged (e.g. ultracentrifuged) after ultrafiltration takes place. Thus, in some embodiments, the invention does not completely replace the use of ultracentrifugation during OMV preparation, but removes at least one step of ultracentrifugation relative to ref. 11. A normal ultracentrifugation step requires about 13 hours for 1.3 litres of OMV suspension, and so a large volume of OMVs requires a large ultracentrifugation resource. Ultrafiltration according to the
10 invention can be used to reduce the volume which has to be ultracentrifuged (by around 3-fold) and so can improve throughput even though ultracentrifugation is not wholly avoided.

The OMVs may be combined with pharmaceutical carriers and/or adjuvants and/or stabilisers. For example, pellet(s) from ultracentrifugation can be re-suspended (e.g. in a sucrose solution, preferably about 3% sucrose) and then subjected to filter sterilisation as described above.

- 15 OMVs may be sonicated. Sonication is particularly useful between re-suspension of centrifugation pellets and sterilisation.

Overall process for preparing sterile OMVs

- In general, therefore, the process of the invention will include the following steps: (1) cultivating bacterial cells; (2) collecting the cultivated cells; (3) OMV formation; (4) separation of OMV from
20 cell debris, to give an aqueous suspension of OMV; (5) ultrafiltration; (6) centrifugation and re-suspension to collect purified OMV; and (7) sterilisation. pH may be adjusted at any stage as required. Similarly, dilution as appropriate can be used.

Step (5) in this process replaces steps (e) and (f) from ref. 11.

The bacterium

- 25 The bacterium from which OMVs are prepared may be Gram-positive, but it is preferably Gram-negative. The bacterium may be from any suitable genus, including *Moraxella* (e.g. *M. catarrhalis* [29,30]), *Shigella* (e.g. *S. flexneri* [31,32]), *Pseudomonas* (e.g. *P. aeruginosa* [31,32]), *Treponema* (e.g. *T. pallidum* [33]), *Haemophilus* (e.g. *H. influenzae* [34 & 35]), *Porphyromonas* (e.g. *P. gingivalis* [36]) or *Helicobacter* (e.g. *H. pylori* [37]), but it is preferably from the *Neisseria* genus. Preferred
30 *Neisseria* species are *N. meningitidis*, *N. lactamica* [38] and *N. gonorrhoeae* [39 & 40]. Within *N. meningitidis*, any of serogroups A, C, W135 and Y may be used, but it is preferred to prepare vesicles from serogroup B. Preferred strains within serogroup B are MC58, 2996, H4476 and 394/98. The best serotypes and strains to use, however, will depend on the strains prevalent in a particular geographical location.

- 35 To reduce pyrogenic activity, it is preferred that the bacterium should have low endotoxin (LPS) levels. Suitable mutant bacteria are known e.g. mutant *Neisseria* [41] and mutant *Helicobacter* [42].

Processes for preparing LPS-depleted outer membranes from Gram-negative bacteria are disclosed in reference 43.

The bacterium may be a wild-type bacterium, or it may be a recombinant bacterium. Preferred recombinant bacteria over-express (relative to the corresponding wild-type strain) immunogens such as NspA, protein 287 [8], protein 741 [8], TbpA, TbpB, superoxide dismutase [7], *etc.* The bacterium may express more than one PorA class I outer membrane protein *e.g.* 2, 3, 4, 5 or 6 of PorA subtypes: P1.7,16; P1.5,2; P1.19,15; P1.5c,10; P1.12,13; and P1.7h,4 [*e.g.* refs. 44 & 45].

Pharmaceutical compositions

For human use, OMVs will generally be combined with a pharmaceutically acceptable carrier.

The term "pharmaceutically acceptable carrier" refers to a carrier for administration of a therapeutic agent, such as antibodies or a polypeptide, genes, and other therapeutic agents. The term refers to any pharmaceutical carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition, and which can be administered without undue toxicity. Suitable carriers can be large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Pharmaceutically acceptable carriers in therapeutic compositions can include liquids such as water, saline, glycerol and ethanol. Auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, can also be present in such vehicles. Typically, the therapeutic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection can also be prepared. Liposomes are included within the definition of a pharmaceutically acceptable carrier. Pharmaceutically acceptable salts can also be present in the pharmaceutical composition, *e.g.* mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in reference 46.

Once formulated, compositions can be administered directly to a subject. Delivery will generally be accomplished by parenteral injection (*e.g.* subcutaneously, intraperitoneally, intravenously or intramuscularly, or to the interstitial space of a tissue) or by mucosal administration (*e.g.* oral, pulmonary, rectal, vaginal, intranasal [47,48]), *etc.*). Transdermal applications, needles, and gene guns or hypodermic sprays may also be used.

The dose and the means of administration of the inventive pharmaceutical compositions are determined based on the specific qualities of the therapeutic composition, the condition, age, and weight of the patient, the progression of the disease, and other relevant factors.

Neisserial infections affect various areas of the body and so the compositions of the invention may be prepared in various forms. For example, the compositions may be prepared as injectables, either as

liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection can also be prepared (e.g. a lyophilised composition). The composition may be prepared for topical administration e.g. as an ointment, cream or powder. The composition be prepared for oral administration e.g. as a tablet or capsule, or as a syrup (optionally flavoured). The composition may be prepared for pulmonary administration e.g. as an inhaler, using a fine powder or a spray. The composition may be prepared as a suppository or pessary. The composition may be prepared for nasal, aural or ocular administration e.g. as drops.

The OMVs of the invention may be combined with an adjuvant. Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to: (A) MF59 (5% Squalene, 0.5% Tween 80, and 0.5% Span 85, formulated into submicron particles using a microfluidizer) [see Chapter 10 of ref. 49; see also ref. 50]; (B) microparticles (i.e. a particle of ~100nm to ~150µm in diameter, more preferably ~200nm to ~30µm in diameter, and most preferably ~500nm to ~10µm in diameter) formed from materials that are biodegradable and non-toxic (e.g. a poly(α-hydroxy acid), a polyhydroxybutyric acid, a polyorthoester, a polyanhydride, a polycaprolactone, etc.), with poly(lactide-co-glycolide) being preferred, optionally treated to have a negatively-charged surface (e.g. with SDS) or a positively-charged surface (e.g. with a cationic detergent, such as CTAB) [51 & 52]; (C) liposomes [see Chapters 13 and 14 of ref. 49]; (D) ISCOMs [see Chapter 23 of ref. 49], which may be devoid of additional detergent [53]; (E) SAF, containing 10% Squalene, 0.4% Tween 80, 5% pluronic-block polymer L121, and thr-MDP, either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion [see Chapter 12 of ref. 49]; (F) Ribi™ adjuvant system (RAS), (Ribi Immunochem) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); (G) saponin adjuvants, such as QuilA or QS21 [see Chapter 22 of ref. 49], also known as Stimulon™; (H) chitosan [e.g. 54]; (I) complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA); (J) cytokines, such as interleukins (e.g. IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, etc.), interferons (e.g. interferon-γ), macrophage colony stimulating factor, tumor necrosis factor, etc. [see Chapters 27 & 28 of ref. 49]; (K) a saponin (e.g. QS21) + 3dMPL + IL-12 (optionally + a sterol) [55]; (L) monophosphoryl lipid A (MPL) or 3-O-deacylated MPL (3dMPL) [e.g. chapter 21 of ref. 49]; (M) combinations of 3dMPL with, for example, QS21 and/or oil-in-water emulsions [56]; (N) oligonucleotides comprising CpG motifs [57] i.e. containing at least one CG dinucleotide; (O) a polyoxyethylene ether or a polyoxyethylene ester [58]; (P) a polyoxyethylene sorbitan ester surfactant in combination with an octoxynol [59] or a polyoxyethylene alkyl ether or ester surfactant in combination with at least one additional non-ionic surfactant such as an octoxynol [60]; (Q) an immunostimulatory oligonucleotide (e.g. a CpG oligonucleotide) and a saponin [61]; (R) an immunostimulant and a particle of metal salt [62]; (S) a saponin and an oil-in-water emulsion [63]; (T) *E.coli* heat-labile enterotoxin ("LT"), or detoxified mutants thereof, such as the K63 or R72 mutants [e.g. Chapter 5 of ref. 64]; (U) cholera toxin ("CT"), or detoxified mutants thereof [e.g.

Chapter 5 of ref. 64]; (V) double-stranded RNA; (W) aluminium salts, such as aluminium hydroxides (including oxyhydroxides), aluminium phosphates (including hydroxyphosphates), aluminium sulfate, etc [Chapters 8 & 9 in ref.49]; (X) monophosphoryl lipid A mimics; such as aminoalkyl glucosaminide phosphate derivatives e.g. RC-529 [65]; (y) polyphosphazene (PCPP); (Z) a bioadhesive [66] such as esterified hyaluronic acid microspheres [67] or a mucoadhesive selected from the group consisting of cross-linked derivatives of poly(acrylic acid), polyvinyl alcohol, polyvinyl pyrrolidone, polysaccharides and carboxymethylcellulose. Other substances that act as immunostimulating agents may also be used [e.g. see Chapter 7 of ref. 49]. Aluminium salts are preferred adjuvants for parenteral immunisation. Mutant toxins are preferred mucosal adjuvants.

10 *OMVs and OMV-based compositions*

The invention provides OMVs obtained by a process of the invention. The invention also provides OMVs obtainable by a process of the invention, which generally comprise outer membrane components in essentially their native form.

15 The invention also provides a composition comprising such OMVs and a pharmaceutically acceptable carrier. The composition may also comprise an adjuvant.

Compositions of the invention are preferably immunogenic compositions, and are more preferably vaccine compositions. Such compositions can be used to raise immune responses (e.g. antibody responses) in a mammal (e.g. in a human, such as a child).

20 The pH of the composition is preferably between 6 and 8, preferably about 7. The pH may be maintained by the use of a buffer. The composition may be sterile and/or pyrogen-free. The composition may be isotonic with respect to humans. The composition may or may not include a preservative (e.g. thiomersal).

Vaccines of the invention may be prophylactic (i.e. to prevent disease) or therapeutic (i.e. to reduce or eliminate the symptoms of a disease).

25 Compositions for administration to patients will comprise an immunologically effective amount of the OMVs. An "immunologically effective amount" is an amount sufficient to effect an immune response in a patient, and more preferably a protective immune response in a patient. The precise amount for a patient will depend upon their size and health, the nature and extent of their condition, and the therapeutics or combination of therapeutics selected for administration. The effective amount
30 for a given situation is determined by routine experimentation and is within the judgment of a physician. For purposes of the present invention, an immunologically effective amount will generally be administered at a dosage of from about 0.01mg/kg to about 5 mg/kg, or about 0.01 mg/kg to about 50 mg/kg or about 0.05 mg/kg to about 10 mg/kg of the composition of the invention in the individual to which it is administered.

35 In addition to OMV antigens, compositions of the invention may include one or more of the following additional antigens:

- a saccharide antigen from *N.meningitidis* serogroup A, C, W135 and/or Y, such as the oligosaccharide disclosed in ref. 68 from serogroup C [see also ref. 69] or the oligosaccharides of ref. 70.
- antigens from *Helicobacter pylori* such as CagA [71 to 74], VacA [75, 76], NAP [77, 78, 79], HopX [e.g. 80], HopY [e.g. 80] and/or urease.
- a saccharide antigen from *Streptococcus pneumoniae* [e.g. 81, 82, 83].
- an antigen from hepatitis A virus, such as inactivated virus [e.g. 84, 85].
- an antigen from hepatitis B virus, such as the surface and/or core antigens [e.g. 85, 86].
- an antigen from hepatitis C virus [e.g. 87].
- a diphtheria antigen, such as a diphtheria toxoid [e.g. chapter 3 of ref. 88].
- a tetanus antigen, such as a tetanus toxoid [e.g. chapter 4 of ref. 88].
- an antigen from *Bordetella pertussis*, such as pertussis holotoxin (PT) and filamentous haemagglutinin (FHA) from *B.pertussis*, optionally also in combination with pertactin and/or agglutinogens 2 and 3 [e.g. refs. 89 & 90]; whole-cell pertussis antigen may also be used.
- a saccharide antigen from *Haemophilus influenzae* B [e.g. 69].
- polio antigen(s) [e.g. 91, 92] such as OPV or, preferably, IPV.
- an antigen from *N.gonorrhoeae* [e.g. 93, 94, 95, 96].
- an antigen from *Chlamydia pneumoniae* [e.g. refs. 97 to 103].
- an antigen from *Porphyromonas gingivalis* [e.g. 104].
- rabies antigen(s) [e.g. 105] such as lyophilised inactivated virus [e.g. 106, RabAvert™].
- measles, mumps and/or rubella antigens [e.g. chapters 9, 10 & 11 of ref. 88].
- influenza antigen(s) [e.g. chapter 19 of ref. 88], such as the haemagglutinin and/or neuraminidase surface proteins.
- antigen(s) from a paramyxovirus such as respiratory syncytial virus (RSV [107, 108]) and/or parainfluenza virus (PIV3 [109]).
- an antigen from *Moraxella catarrhalis* [e.g. 110].
- an antigen from *Streptococcus pyogenes* (group A streptococcus) [e.g. 111, 112, 113].
- an antigen from *Staphylococcus aureus* [e.g. 114].
- an antigen from *Bacillus anthracis* [e.g. 115, 116, 117].
- an antigen from a virus in the flaviviridae family (genus flavivirus), such as from yellow fever virus, Japanese encephalitis virus, four serotypes of Dengue viruses, tick-borne encephalitis virus, West Nile virus.
- a pestivirus antigen, such as from classical porcine fever virus, bovine viral diarrhoea virus, and/or border disease virus.
- a parvovirus antigen e.g. from parvovirus B19.
- a prion protein (e.g. the CJD prion protein)
- an amyloid protein, such as a beta peptide [118]
- a cancer antigen, such as those listed in Table 1 of ref. 119 or in tables 3 & 4 of ref. 120.

The inclusion of further *N.meningitidis* antigens is preferred.

Where a saccharide or carbohydrate antigen is used, it is preferably conjugated to a carrier protein in order to enhance immunogenicity [e.g. refs. 121 to 130]. Preferred carrier proteins are bacterial toxins or toxoids, such as diphtheria or tetanus toxoids. The CRM₁₉₇ diphtheria toxoid is particularly preferred [131]. Other carrier polypeptides include the *N.meningitidis* outer membrane protein [132], synthetic peptides [133, 134], heat shock proteins [135, 136], pertussis proteins [137, 138], protein D from *H.influenzae* [139], cytokines [140], lymphokines [140], hormones [140], growth factors [140], toxin A or B from *C.difficile* [141], iron-uptake proteins [142], etc. Different saccharides can be conjugated to the same or different type of carrier protein. Any suitable conjugation reaction can be used, with any suitable linker where necessary.

Toxic protein antigens may be detoxified where necessary e.g. detoxification of pertussis toxin by chemical and/or genetic means [90].

Where a diphtheria antigen is included in the composition it is preferred also to include tetanus antigen and pertussis antigens. Similarly, where a tetanus antigen is included it is preferred also to include diphtheria and pertussis antigens. Similarly, where a pertussis antigen is included it is preferred also to include diphtheria and tetanus antigens. DTP combinations are thus preferred.

Antigens in the composition will typically be present at a concentration of at least 1µg/ml each. In general, the concentration of any given antigen will be sufficient to elicit an immune response against that antigen.

As an alternative to using protein antigens in the composition of the invention, nucleic acid encoding the antigen may be used [e.g. refs. 143 to 151]. Protein components of the compositions of the invention may thus be replaced by nucleic acid (preferably DNA e.g. in the form of a plasmid) that encodes the protein.

Methods of treatment

The invention provides a method for raising an immune response in a patient, comprising administering an immunogenic dose of OMVs of the invention to the patient. The immune response is preferably protective and preferably involves antibodies and/or cell-mediated immunity. The method may raise a booster response.

The patient is preferably a human. Where the vaccine is for prophylactic use, the human is preferably a child (e.g. a toddler or infant) or a teenager; where the vaccine is for therapeutic use, the human is preferably a teenager or an adult. A vaccine intended for children may also be administered to adults e.g. to assess safety, dosage, immunogenicity, etc.

The invention also provides OMVs of the invention for use in medicine.

The invention also provides the use of OMVs of the invention in the manufacture of a medicament for treating and/or preventing meningococcal infection and/or bacterial meningitis.

The invention may be used to elicit systemic and/or mucosal immunity.

Dosage treatment can be a single dose schedule or a multiple dose schedule. Multiple doses may be used in a primary immunisation schedule and/or in a booster immunisation schedule.

Definitions

5 The term "OMV" includes any proteoliposomic vesicle obtained by disrupting a bacterial outer membrane to form vesicles of the outer membrane which include protein components of the outer membrane. OMVs are prepared artificially from bacteria (e.g. by detergent treatment) and are thus distinct from microvesicles (MVs [152]) and 'native OMVs' ('NOMVs' [48]), both of which are naturally-occurring membrane vesicles that form spontaneously during bacterial growth and are released into culture medium. MVs can be obtained by culturing *Neisseria* in broth culture medium, separating whole cells from the smaller blebs in the broth culture medium, and then collecting the MVs from the cell-depleted medium. Strains for use in production of MVs can generally be selected on the basis of the amount of MVs produced in culture e.g. refs. 153 & 154 describe *Neisseria* with high MV production.

15 The term "comprising" means "including" as well as "consisting" e.g. a composition "comprising" X may consist exclusively of X or may include something additional e.g. X + Y.

The term "about" in relation to a numerical value x means, for example, $x \pm 10\%$.

The word "substantially" does not exclude "completely" e.g. a composition which is "substantially free" from Y may be completely free from Y. Where necessary, the word "substantially" may be omitted from the definition of the invention.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows a process including two ultracentrifugation steps without ultrafiltration. Figure 2 shows a process in which one ultracentrifugation step has been replaced by an ultrafiltration step.

MODES FOR CARRYING OUT THE INVENTION

25 Example 1 — OMVs from meningococcal serogroup B

N.meningitidis serogroup B (strain 44/76, from Norway) was cultured on eight "selective medium for Meningococci" plates at 35°C in 5% CO₂/air atmosphere for 24 hours. Cells were harvested into 2 tubes with 12 ml Frantz' medium. Contents of tubes were added to 2x500ml flasks containing Frantz' medium (150ml) and grown with shaking for 12 hours to obtain the correct growth for transferring into 2x500ml flasks containing Frantz' medium (1500ml). The flasks were grown with shaking for a further 12 hours to yield the inoculum. One flask was added to a Chemap fermentor with 300L capacity, containing 110L of pre-sterilised Frantz' medium and sterile-filtered dialysed yeast extract. The pH after inoculation was 7.1, maintained at 7.0 with 3N NaOH. A surface aeration fermentation was performed, controlling the amount of air O₂ and stirrer applied, and cultivating for

10 hours at 35°C. Growth was terminated at an OD_{590nm} of 7.10, the fermentor was cooled under 15°C, the air supply was reduced and stirring continued at 100 rpm overnight.

Transfer of the bacterial suspension from the fermentor was done by pressure to a Millipore CUF cross flow filtration unit equipped with valves, pumps and a filter module with 4 Pellicon P2B300V05 polyethersulphone filters (300kD cutoff). Initial transfer of 30L bacterial suspension was followed with a constant volume concentration until the fermentor was emptied, and then a further concentration was performed to give a volume of 5.5L.

Concentrating the suspension was performed in the CFF unit by circulating the suspension to be passing by the filters, with a transmembrane pressure being continuously monitored and kept less than 0.5 bar (observed: 0.5 bar at the end of concentration).

Adjustment of pH of the concentrated bacterial suspension from pH 7.0 to 8.2 was done by adding, via a tubing system, 5L of 0.1 M Tris-HCl buffer of pH 9 with 10 mM EDTA, followed by 15 min stirring in the CFF unit to secure uniform conditions.

Inactivation/extraction of outer membrane (OM) material was initiated by adding, via tubing, 500 ml of an 0.1M Tris-HCl buffer (pH 9) containing 10% deoxycholate (DOC), to give a final concentration of 0.5%. Subsequently the suspension was circulated in the CFF unit for 30 min, and the extracted suspension (9.5L), checked to be completely without living bacteria, was drained off by pumping into a 25L bottle.

In a first experiment (experiment A; Figure 1), crude OMVs were prepared by distributing the inactivated suspension to centrifuge tubes of 500ml, and centrifuging in a Beckman centrifuge at 9000 rpm (16650 x g) for 1 hour at 4°C, collecting 8.5L of supernatant. 1.35L of crude OMVs was purified by two subsequent ultracentrifugations at 19000 rpm, 4°C, for 13.6 hours and 6.8 hours respectively, collecting the pellet. The pellet was suspended in 660ml of 3% sucrose with magnetic stirring at room temperature until homogeneous, obtaining a concentration of the purified material of 1.52g/L of total protein.

In a second experiment (experiment B; Figure 2), 3L of crude OMVs were prepared from bacterial suspension using a CUF cross flow filtration unit equipped with valves, pumps and a filter module with 1 Pellicon P2B300V05 polyethersulphone filters (300kD cutoff). Initial transfer of 1L crude OMVs was followed with a constant volume concentration until the 3L was finished, and then diafiltered by adding 5L of 0.05 M Tris-HCl buffer of pH 8.6 with 2 mM EDTA, 1% of DOC and 20% sucrose. The retentate obtained was purified by ultracentrifugation at 19000rpm, 4°C, for 6,8 hours, collecting the pellet. The pellet was suspended in 1L of 3% sucrose with magnetic stirring at room temperature until homogeneous, obtaining a concentration of 0.85g/L total protein in the purified material.

Final purifications of OMV obtained by both experiment A and B, after a dilution with 3% sucrose around 1.2g/L of total protein, were both performed at 20°C by filtering through 3 capsule filters

(Gelman Science Suporlife DCF) in sequence, first pre filters of 0.8 μ m and 0.45 μ m, respectively, then the final sterile filtration (0.22 μ m), testing 836ml of purified material for experiment A, with an initial protein concentration of 1.1 mg/ml, and 1L for experiment B. The OMV protein concentrations after the filtration were 0.12 mg/ml and 0.59 mg/ml respectively.

5 OMVs were characterised as follows:

| | Experiment A | Experiment B | Specification |
|--------------------------------------|-------------------|-------------------|--------------------|
| Deoxycholate (μ g/g protein) | 1.5 | 0.4 | 0.1 – 0.4 |
| DNA (μ g/g protein) | 0.004 | 0.004 | < 0.035 |
| Endotoxin (UI/g protein) | 2.8×10^3 | 2.6×10^3 | < 20×10^3 |
| LPS (μ g/g protein) | 0.05 | 0.08 | 0.06 – 0.12 |
| SDS page | | | |
| 80kDa | 1.7 | 2.2 | 1 – 4 |
| 70kDa | 11.8 | 12.7 | 1 – 12 |
| class I | 24.6 | 25.1 | 22 – 32 |
| class III | 34.8 | 32.8 | 30 – 43 |
| class IV | 12.0 | 12.2 | 9 – 18 |
| class V | 15.0 | 15.1 | 10 – 24 |

Thus the OMVs prepared using ultrafiltration have a similar composition to those obtained by ultracentrifugation. In comparison to the prior art method, however, the method of the invention is much simpler and quicker.

10 *Example 2 — OMVs from meningococcal serogroup B*

N.meningitidis serogroup B (strain NZ 98/254, from New Zealand) was cultured as before, except that: (a) Catlin medium was used in place of Frantz' medium; (b) the initial 150ml cultures were grown to a level ready for transferring into a Chemap fermentor with 300L capacity, containing 120L of pre-sterilized medium; (c) growth in the Chemap fermentor was for 12 hours; (d) growth was
15 terminated at OD_{590nm} of 5.90.

Transfer from the fermentor was as before, except that concentration was performed until 5L volume. Concentration was performed as before.

pH was adjusted as before, except that: (a) the final pH was 8.6; (b) the amount of 0.1 M Tris-HCl buffer added was 6L.

20 Inactivation/extraction was as before, except: (a) 600 ml of the Tris-HCl buffer was added; (b) the volume of extracted suspension was 19.5L.

Preparation of crude OMVs was as before, except: (a) centrifuge tubes were 1000ml volume; (b) centrifugation was at 8000 rpm (16650 x g), to give 17.5L of supernatant.

5 Cross-flow filtration for purifying OMVs (in place of centrifugation) was as in experiment B above, except: (a) using 17.5L crude OMVs; (b) using two P2B300V05 polyethersulphone filters (300 cutoff); (c) using an initial transfer of 4L crude OMVs; (d) diafiltration with 30L Tris-HCl buffer; (e) pellet was resuspended in 1.2L of 3% sucrose; (f) the homogenous material was further sonicated, and gave a final concentration of purified material of 1.5g/L total protein.

Final purification was as before, except: (a) filtration was through two capsule filters (Sartoclean CA, Sartobran P) in sequence, first pre-filters of 0.8+0.65 μm , then a final sterile filtration 0.45+0.22 μm . The OMV protein concentration after the filtration was 1.0 g/L.

OMVs were characterised as follows:

| | Example 2 | Specification |
|---|-----------|--------------------|
| Deoxycholate ($\mu\text{g/g}$ protein) | 0.4 | 0.1 – 0.4 |
| DNA ($\mu\text{g/g}$ protein) | 0.0005 | < 0.035 |
| Endotoxin (UI/g protein) | 5393 | < 20×10^3 |
| LPS ($\mu\text{g/g}$ protein) | 0.10 | 0.06 – 0.12 |
| SDS page | | |
| 80kDa | 3.8 | 1 – 4 |
| 70kDa | 6.4 | 1 – 12 |
| class I | 18.7 | 22 – 32 |
| class III+FbpA | 31.3 | 30 – 43 |
| class IV | 10.7 | 9 – 18 |
| class V | 2.7 | 10 – 24 |
| NspA | 3.9 | 1 – 7 |

10

Thus the production method provided OMVs with a native antigen mosaic and a strongly reduced level of LPS. In comparison to the prior art method where two ultracentrifugation steps are used, however, the invention is much simpler and quicker.

15

It will be understood that the invention has been described by way of example only and modifications may be made whilst remaining within the scope and spirit of the invention.

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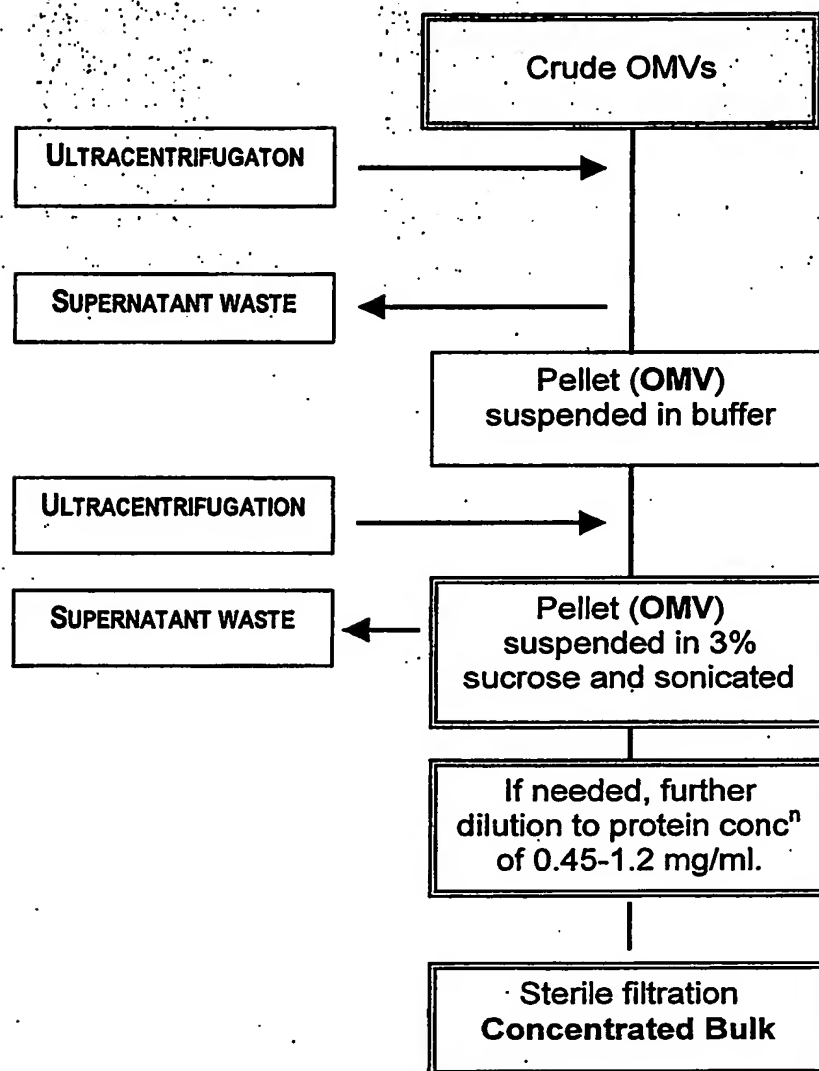
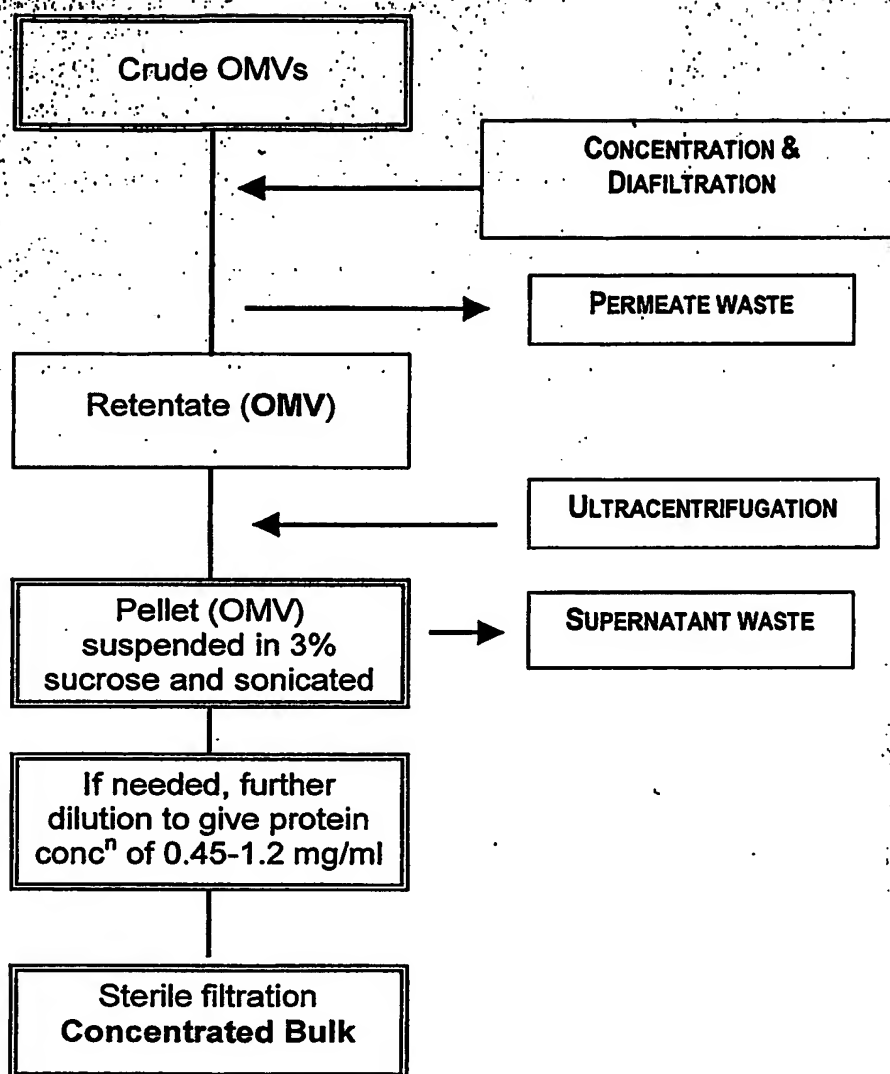
FIGURE 1

FIGURE 2

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